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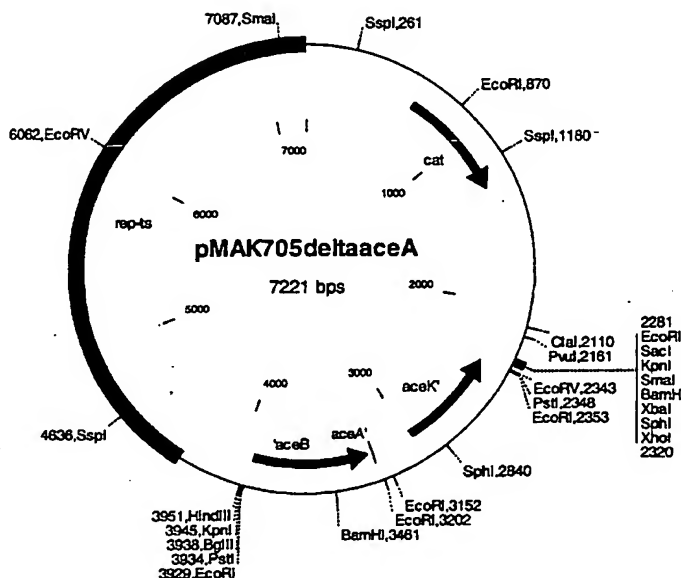
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(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTE-  
RIACEAE THAT CONTAIN AN ATTENUATED ACEA GENE



(57) Abstract: The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the fol-  
lowing steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-  
amino acid, in which the aceA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off; b) enrichment of  
the L-amino acid in the medium or in the cells of the bacteria; and c) isolation of the L-amino acid.

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**Process for the Production of L-Amino Acids using  
Strains of the Family Enterobacteriaceae that contain  
an Attenuated aceA Gene**

Field of the Invention

- 5 The present invention relates to a process for the enzymatic production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae in which the aceA gene is attenuated.

Prior Art

- 10 L-amino acids, in particular L-threonine, are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry, and most especially in animal nutrition.

- It is known to produce L-amino acids by fermentation of  
15 strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. On account of their great importance efforts are constantly being made to improve processes for producing the latter. Process improvements may relate to fermentation technology  
20 measures, such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during the fermentation, or the working-up to the product form, for example by ion exchange chromatography, or the intrinsic  
25 performance properties of the microorganism itself.

- Methods comprising mutagenesis, selection and mutant choice are employed in order to improve the performance properties of these microorganisms. In this way strains are obtained that are resistant to antimetabolites, such as for example  
30 the threonine analogue  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV) or are auxotrophic for regulatorily important metabolites, and that produce L-amino acids such as for example L-threonine.

Methods of recombinant DNA technology have also been used for some years in order to improve strains of the family Enterobacteriaceae producing L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating  
5 their effect on production.

#### Object of the Invention

The object of the invention is to provide new measures for the improved enzymatic production of L-amino acids, in particular L-threonine.

#### 10 Summary of the Invention

The invention provides a process for the enzymatic production of L-amino acids, in particular L-threonine, using microorganisms of the family Enterobacteriaceae that in particular already produce L-amino acids and in which  
15 the nucleotide sequence coding for the aceA gene is attenuated.

#### Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned hereinafter, this is understood to mean one or more amino  
20 acids including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and  
25 L-arginine. L-threonine is particularly preferred.

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by using for example a weak  
30 promoter or a gene or allele that codes for a corresponding enzyme with a low activity and/or that inactivates the

corresponding enzyme (protein) or gene, and optionally combining these measures.

By means of these attenuation measures the activity or concentration of the corresponding protein is generally  
5 reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild type protein, or the activity or concentration of the protein in the initial microorganism.

The process is characterized in that the following steps  
10 are carried out:

- a) fermentation of microorganisms of the family Enterobacteriaceae in which the aceA gene is attenuated,
- b) enrichment of the corresponding L-amino acid in the  
15 medium or in the cells of the microorganisms of the family Enterobacteriaceae, and
- c) isolation of the desired L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or portions  
20 thereof remain in the product.

The microorganisms that are the subject of the present invention can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. The  
25 microorganisms are members of the family Enterobacteriaceae selected from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. In the case of the genus Escherichia the species Escherichia coli may in particular be mentioned,  
30 and in the case of the genus Serratia the species Serratia marcescens may in particular be mentioned.

Suitable strains of the genus *Escherichia*, in particular those of the species *Escherichia coli*, that produce in particular L-threonine, include for example:

- Escherichia coli TF427
- 5 Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIGenetika MG442
- Escherichia coli VNIIGenetika M1
- Escherichia coli VNIIGenetika 472T23
- 10 Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132

Suitable strains of the genus *Serratia*, in particular of the species *Serratia marcescens*, that produce L-threonine  
15 include for example:

- Serratia marcescens* HNr21
- Serratia marcescens* TLR156
- Serratia marcescens* T2000

Strains of the family of Enterobacteriaceae producing  
20 L-threonine preferably have, *inter alia*, one or more of the genetic or phenotype features selected from the following group: resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic  
25 acid, resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues such as for example valine hydroxamate, resistance to purine analogues such as for example 6-dimethylaminopurine, need for L-methionine, optionally  
30 partial and compensable need for L-isoleucine, need for meso-diaminopimelic acid, auxotrophy with regard to threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid,

resistance to L-aspartate, resistance to L-leucine,  
resistance to L-phenylalanine, resistance to L-serine,  
resistance to L-cysteine, resistance to L-valine,  
sensitivity to fluoropyruvate, defective threonine  
5 dehydrogenase, optionally ability to utilize sucrose,  
enhancement of the threonine operon, enhancement of  
homoserine dehydrogenase, I-aspartate kinase I, preferably  
of the feedback-resistant form, enhancement of homoserine  
kinase, enhancement of threonine synthase, enhancement of  
10 aspartate kinase, optionally of the feedback-resistant  
form, enhancement of aspartate semialdehyde dehydrogenase,  
enhancement of phosphoenol pyruvate carboxylase, optionally  
of the feedback-resistant form, enhancement of phosphoenol  
pyruvate synthase, enhancement of transhydrogenase,  
15 enhancement of the RhtB gene product, enhancement of the  
RhtC gene product, enhancement of the YfiK gene product,  
enhancement of a pyruvate carboxylase, and attenuation of  
acetic acid formation.

It has now been found that microorganisms of the family  
20 Enterobacteriaceae after attenuation, in particular after  
switching off the aceA gene, produce L-amino acids, in  
particular L-threonine, in an improved way.

The nucleotide sequences of the Escherichia coli genes  
belong to the prior art and may also be obtained from the  
25 genome sequence of Escherichia coli published by Blattner  
et al. (Science 277, 1453 - 1462 (1997)).

The aceA gene is described *inter alia* by the following  
data:

Designation:	Isocitrate lyase
30 EC-No.:	4.1.3.1
Reference:	Matsuoko and McFadden; Journal of Bacteriology 170, 4528-4536 (1988)
Accession No.:	AE000474

Apart from the described *aceA* gene, alleles of the gene may be used that result from the degeneracy of the genetic code or from functionally neutral sense mutations, the activity of the protein not being substantially altered.

- 5 In order to achieve an attenuation the expression of the gene or the catalytic properties of the enzyme proteins may for example be reduced or switched off. Optionally both measures may be combined.

The gene expression may be reduced by suitable culture  
10 conditions, by genetic alteration (mutation) of the signal structures of the gene expression, or also by antisense-RNA techniques. Signal structures of the gene expression are for example repressor genes, activator genes, operators, promoters, attenuators, ribosome-binding sites, the start  
15 codon and terminators. The person skilled in the art may find relevant information in, *inter alia*, articles by Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), by Carrier and Keasling (Biotechnology Progress 15, 58-64 (1999), Franch and Gerdes (Current  
20 Opinion in Microbiology 3, 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6<sup>th</sup> Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene and Klone", VCH  
25 Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change or reduction of the catalytic properties of enzyme proteins are known from the prior art. As examples there may be mentioned the work by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-  
30 8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences, USA 95, 5511-5515 (1998), Wentz and Schachmann (Journal of Biological Chemistry 266, 20833-20839 (1991)). Detailed information may be obtained from known textbooks on genetics and molecular biology, such as

for example that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations include transitions, transversions, insertions and deletions. Depending on the action of the amino acid exchange on the enzyme activity, one speaks of missense mutations or nonsense mutations. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations, which in turn lead to the incorporation of false amino acids or the premature termination of a translation. If as a result of the mutation a stop codon is formed in the coding region, this also leads to a premature termination of the translation. Deletions of several codons typically lead to a complete disruption of the enzyme activity. Details regarding the production of such mutations belong to the prior art and may be obtained from known textbooks on genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6<sup>th</sup> Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations in the genes such as for example deletion mutations may be incorporated by gene and/or allele exchange in suitable strains.

A conventional method is the method of gene exchange by means of a conditionally replicating pSC101 derivate pMAK705 described by Hamilton et al. (Journal of Bacteriology 171, 4617 - 4622 (1989)). Other methods described in the prior art, such as for example that of Martinez-Morales et al. (Journal of Bacteriology 181, 7143-7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182, 842-847 (2000)) may likewise be used.



It is also possible to transfer mutations in the respective genes or mutations relating to the expression of the relevant genes, by conjugation or transduction into various strains.

- 5 Furthermore for the production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae it may be advantageous in addition to the attenuation of the aceA gene also to enhance one or more enzymes of the known threonine biosynthesis pathway or  
10 enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide-adenine-dinucleotide phosphate.

The term "enhancement" describes in this connection the raising of the intracellular activity of one or more  
15 enzymes or proteins in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes, using a strong promoter or a gene that codes for a corresponding enzyme or protein having a high activity, and optionally by combining these  
20 measures.

By means of the enhancement measures, in particular overexpression, the activity or concentration of the corresponding protein is in general raised by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at  
25 most up to 1000% or 2000% referred to that of the wild type protein and/or the activity or concentration of the protein in the initial microorganism.

Thus, one or more of the genes selected from the following group may for example be simultaneously enhanced, in  
30 particular overexpressed:

- the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),

- the *pyc* gene coding for pyruvate carboxylase (DE-A-19 831 609),
- the *pps* gene coding for phosphoenol pyruvate synthase (Molecular and General Genetics 231:332 (1992)),
- 5 • the *ppc* gene coding for phosphoenol pyruvate carboxylase (Gene 31:279-283 (1984)),
- the genes *pntA* and *pntB* coding for transhydrogenase (European Journal of Biochemistry 158:647-653 (1986)),
- the gene *rhtB* imparting homoserine resistance (EP-A-0 994 190),
- 10 • the *mgo* gene coding for malate:quinone oxidoreductase (DE 100 348 33.5),
- the gene *rhtC* imparting threonine resistance (EP-A-1 013 765), and
- 15 • the *thrE* gene of *Corynebacterium glutamicum* coding for threonine export (DE 100 264 94.8).

The use of endogenous genes is in general preferred. The term "endogenous genes" or "endogenous nucleotide sequences" is understood to mean the genes or nucleotide sequences  
20 present in the population of a species.

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition to the attenuation of the *aceA* gene also to attenuate, in particular to switch off or reduce the expression of one or  
25 more of the genes selected from the following group:

- the *tdh* gene coding for threonine dehydrogenase (Ravnikar and Somerville, Journal of Bacteriology 169, 4716-4721 (1987)),

- the mdh gene coding for malate dehydrogenase (E.C. 1.1.1.37) (Vogel et al., Archives in Microbiology 149, 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfa  
5 (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA),
- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 des National Center for Biotechnology Information (NCBI, Bethesda, MD, USA),
- 10 • the pckA gene coding for the enzyme phosphoenol pyruvate carboxykinase (Medina et al. (Journal of Bacteriology 172, 7151-7156 (1990)),
- the poxB gene coding for pyruvate oxidase (Grabau and Cronan (Nucleic Acids Research 14 (13), 5449-5460  
15 (1986)),
- the dgsA gene coding for the regulator of the phosphotransferase system (Hosono et al., Bioscience, Biotechnology and Biochemistry 59, 256-251 (1995) and Accession No.: AE000255), and
- 20 • the fruR gene coding for the fructose repressor (Jahreis et al., Molecular and General Genetics 226, 332-336 (1991) and Accession No.: AE000118)

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition  
25 to the attenuation of the aceA gene also to switch off undesirable secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

30 The microorganisms produced according to the invention may be cultivated in a batch process (batch cultivation), in a

fed batch process (feed process) or in a repeated fed batch process (repetitive feed process). A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die

5 Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Störhas (Bioreaktoren and periphere Einrichtungen (Vieweg Verlag, Brunswick /Wiesbaden, 1994)).

The culture medium to be used must appropriately satisfy

10 the requirements of the respective strains. Descriptions of culture media of various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

15 As carbon sources, sugars and carbohydrates such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats such as for example soya bean oil, sunflower oil, groundnut oil and coconut oil, fatty acids such as for example

20 palmitic acid, stearic acid and linoleic acid, alcohols such as for example glycerol and ethanol, and organic acids such as for example acetic acid, may be used. These substances may be used individually or as a mixture.

As nitrogen source, organic nitrogen-containing compounds

25 such as peptones, yeast extract, meat extract, malt extract, maize starch water, soya bean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate may be used. The nitrogen sources may be

30 used individually or as a mixture.

As phosphorus source, phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts may be used. The culture medium may furthermore contain traces of metals,

- such as for example magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth promoters such as amino acids and vitamins may be used in addition to the aforementioned substances. Apart from  
5 these, suitable precursors may be added to the culture medium. The aforementioned starting substances may be added to the culture in the form of a single batch or may be metered in in an appropriate manner during the cultivation.
- 10 In order to regulate the pH of the culture basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds such as phosphoric acid or sulfuric acid are used as appropriate. In order to control foam formation antifoaming agents such as for  
15 example fatty acid polyglycol esters may be used. In order to maintain the stability of plasmids, suitable selectively acting substances, for example antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures such as for  
20 example air are fed into the culture. The temperature of the culture is normally 25°C to 45°C, and preferably 30°C to 40°C. Cultivation is continued until a maximum amount of L-amino acids (or L-threonine) has been formed. This target is normally achieved within 10 hours to 160 hours.
- 25 The L-amino acids may be analyzed by anion exchange chromatography followed by ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-  
30 1174).

The process according to the invention can be used for the enzymatic production of L-amino acids, such as for example L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

A pure culture of the Escherichia coli K-12 strain DH5 $\alpha$ /pMAK705 was filed as DSM 13720 on 08 September 2000 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) according to the Budapest  
 5 Convention.

The present invention is described in more detail hereinafter with the aid of examples of implementation.

The isolation of plasmid DNA from Escherichia coli as well as all techniques for the restriction, Klenow treatment and  
 10 alkaline phosphatase treatment are carried out according to Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold-Spring Harbor Laboratory Press). The transformation of Escherichia coli is, unless otherwise described, carried out according to Chung et al.  
 15 (Proceedings of the National Academy of Sciences of the United States of America, USA (1989) 86: 2172-2175).

The incubation temperature in the production of strains and transformants is 37°C. In the gene exchange process according to Hamilton et al., temperatures of 30°C and 44°C  
 20 are used.

#### Example 1

Construction of the deletion mutation of the aceA gene.

Parts of the gene regions lying upstream and downstream of the aceA gene are amplified from Escherichia coli K12 using  
 25 the polymerase chain reaction (PCR) as well as synthetic oligonucleotides. Starting from the nucleotide sequence of the aceBAK operon in E. coli K12 MG1655 DNA (SEQ ID No. 1) the following PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

30 aceA'5'-1: 5' - ATGCTTACTCACGCCTGTTG - 3' (SEQ ID No. 3)

aceA'3'-1: 5' - CATTCTTACTGCTGTTG - 3' (SEQ ID No. 4)

aceA'3'-1: 5' - CAACAACAACCGTTGCTGAC - 3' (SEQ ID No. 5)

aceA'3'-2: 5' - CAGTTCGTTCCGCCACCTGTA - 3' (SEQ ID No. 6)

The chromosomal E. coli K12 MG1655 DNA used for the PCR is isolated according to the manufacturer's instructions using

5 "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A ca. 700 bp large DNA fragment from the region lying upstream of the aceA gene (designated 'aceB') and a ca. 800 bp large DNA fragment from the region lying downstream of the aceA gene (designated aceK') can be amplified with

10 the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with Taq DNA polymerase (Gibco-BRL, Eggenstein, Germany). The PCR products are ligated according to the manufacturer's instructions in

15 each case with the vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, Netherlands) and transformed in the E. coli strain TOP10F'. The selection of plasmid-carrying cells is carried out on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA isolation

20 the vector pCR2.1TOPO'aceB is cleaved with the restriction enzymes EcoRV and SpeI, and the 'aceB fragment after separation in 0.8% agarose gel is isolated using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After the plasmid DNA isolation the vector pCR2.1TOPOaceK'

25 is cleaved with the enzymes Ecl136II and SpeI and ligated with the isolated 'aceB fragment. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA

30 isolation, those plasmids in which the mutagenic DNA sequence shown in SEQ ID No. 7 is present in cloned form are detected by control cleavage with the enzymes HindIII and XbaI. One of the plasmids is designated pCR2.1TOPOΔaceA.

Example 2Construction of the exchange vector pMAK705 $\Delta$ aceA

The aceBAK allele described in Example 1 is isolated from the vector pCR2.1TOPO $\Delta$ aceA after restriction with the  
5 enzymes HindIII and XbaI and separation in 0.8% agarose gel, and is ligated with the plasmid pMAK705 (Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622), that had been digested with the enzymes HindIII and XbaI. The ligation batch is transformed in DH5 $\alpha$  and plasmid-carrying  
10 cells are selected on LB agar to which 20  $\mu$ g/ml of chloramphenicol have been added. The successful cloning is detected after plasmid DNA isolation and cleavage with the enzymes BamHI, KpnI, SphI, SpeI and PstI. The resultant exchange vector pMAK705 $\Delta$ aceA (= pMAK705 $\Delta$ aceA) is shown  
15 in Fig. 1.

Example 3

## Site-specific mutagenesis of the aceA gene in the E. coli strain MG442

The E. coli strain MG442 producing L-threonine is described  
20 in patent specification US-A- 4,278,765 and is filed as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

For the exchange of the chromosomal aceA gene by the plasmid-coded deletion construct, MG442 is transformed with  
25 the plasmid pMAK705 $\Delta$ aceA. The gene exchange is carried out by the selection process described by Hamilton et al.

(1989) Journal of Bacteriology 171, 4617 - 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic  
30 Press) with the following oligonucleotide primers:

aceA'5'-1: 5' - ATGCTTACTCACGCCTGTTG - 3' (SEQ ID No. 3)



aceA'3'-2: 5' - CAGTTCGTTCCGCCACCTGTA - 3' (SEQ ID No. 6)

The resultant strain is designated MG442ΔaceA.

#### Example 4

Production of L-threonine using the strain MG442ΔaceA

- 5 MG442ΔaceA is cultivated on minimal medium having the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose and 20 g/l agar. The formation of L-threonine is checked in batch cultures of 10 ml that are contained in 100 ml Erlenmeyer
- 10 flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose are inoculated and incubated for 16 hours at 37°C and 180 rpm in an ESR incubator from Kühner AG
- 15 (Birsfelden, Switzerland). 250 µl of this preculture are reinoculated in 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$  and 20 g/l glucose) and incubated for 48 hours at 37°C. After incubation the
- 20 optical density (OD) of the culture suspension is measured with an LP2W photometer from the Dr. Lange company (Dusseldorf, Germany) at a measurement wavelength of 660 nm.

- The concentration of formed L-threonine is then determined
- 25 in the sterile-filtered culture supernatant using an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the test is given in Table 1.

Table 1

Strain	OD (660 nm)	L-threonine g/l
MG442	6.0	1.5
MG442 $\Delta$ aceA	6.2	1.9

Brief Description of the Figure:

- Fig. 1: pMAK705 $\Delta$ aceA ( = pMAK705 $\Delta$ aceA)

5 Length data are given as approximate values. The abbreviations and acronyms used have the following meanings:

- cat: chloramphenicol resistance gene
- rep-ts: temperature-sensitive replication region of  
10 the plasmid pSC101
- 'aceB: part of the 3' region of the aceB gene
- aceA': ATG start codon of the aceA gene
- aceK': part of the 5' region of the aceK gene

The abbreviations for the restriction enzymes have the  
15 following meanings:

- BamHI: restriction endonuclease from *Bacillus amyloliquefaciens*
- BglII: restriction endonuclease from *Bacillus globigii*
- 20 • ClaI: restriction endonuclease from *Caryophanon latum*
- EcoRI: restriction endonuclease from *Escherichia coli*

- EcoRV: restriction endonuclease from *Escherichia coli*
- HindIII: restriction endonuclease from *Haemophilus influenzae*
- 5 • KpnI: restriction endonuclease from *Klebsiella pneumoniae*
- PstI: restriction endonuclease from *Providencia stuartii*
- PvuI: restriction endonuclease from *Proteus vulgaris*
- 10 • SacI: restriction endonuclease from *Streptomyces achromogenes*
- SalI: restriction endonuclease from *Streptomyces albus*
- SmaI: restriction endonuclease from *Serratia marcescens*
- 15 • SphI: restriction endonuclease from *Streptomyces phaeochromogenes*
- SspI: restriction endonuclease from *Sphaerotilus species*
- 20 • XbaI: restriction endonuclease from *Xanthomonas badrii*
- XhoI: restriction endonuclease from *Xanthomonas holcicola*

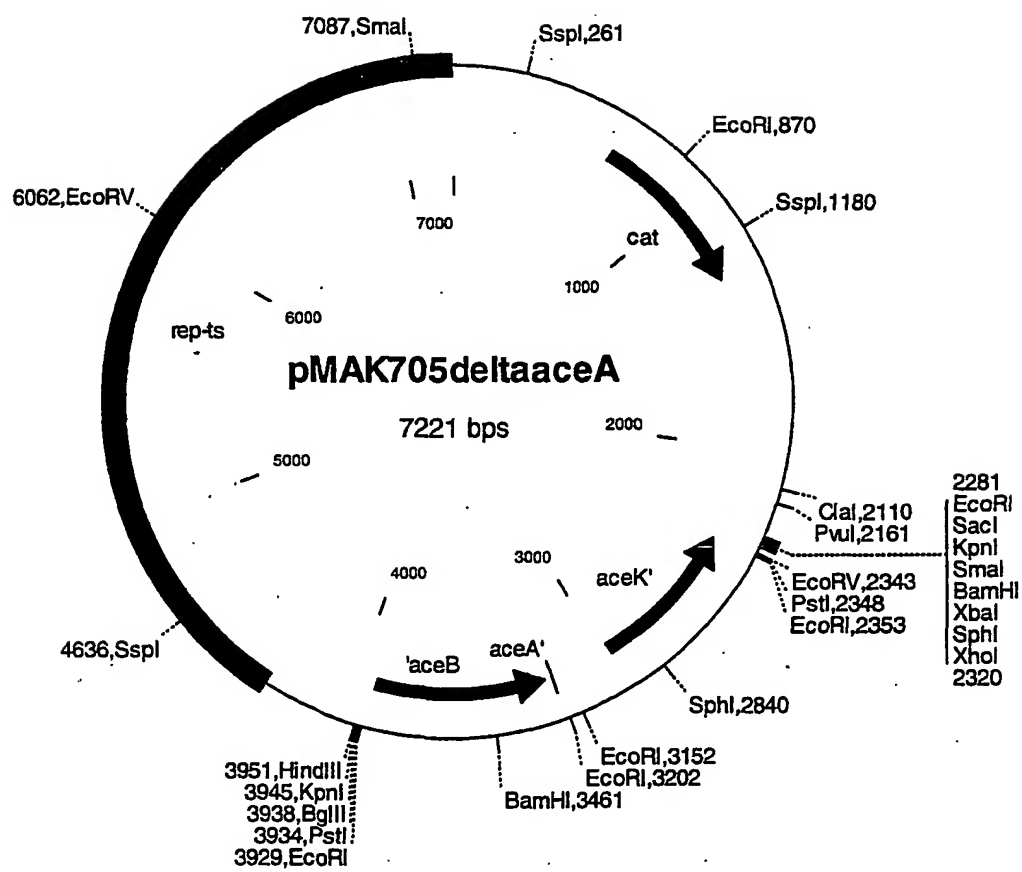
**What is Claimed is:**

1. Process for the production of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
  - 5 a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the aceA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off,
  - 10 b) enrichment of the L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or portions  
15 thereof remain in the product.
2. Process according to claim 1, wherein microorganisms are used in which in addition further genes of the biosynthesis pathway of the desired L-amino acid are enhanced.
- 20 3. Process according to claim 1, wherein microorganisms are used in which the metabolic pathways that reduce the formation of the desired L-amino acid are at least partially switched off.
4. Process according to claim 1, wherein the expression  
25 of the polynucleotide(s) that codes/code for the aceA gene is attenuated, in particular is switched off.
5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide aceA codes are  
30 reduced.

6. Process according to claim 1, wherein, for the production of L-amino acids, microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is enhanced, in particular overexpressed:
- 5 6.1 the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 10 6.2 the pyc gene coding for pyruvate carboxylase,
- 6.3 the pps gene coding for phosphoenol pyruvate synthase,
- 6.4 the ppc gene coding for phosphoenol pyruvate carboxylase,
- 15 6.5 the genes pntA and pntB coding for transhydrogenase,
- 6.6 the gene rhtB imparting homoserine resistance,
- 6.7 the mgo gene coding for malate:quinone oxidoreductase,
- 20 6.8 the gene rhtC imparting threonine resistance, and
- 6.9 the thrE gene coding for threonine export.
7. Process according to claim 1, wherein, for the production of L-amino acids, microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is attenuated, in particular switched off, or the expression is reduced:
- 25 7.1 the tdh gene coding for threonine dehydrogenase,
- 7.2 the mldh gene coding for malate dehydrogenase,

- 7.3 the gene product of the open reading frame (orf) yjfa,
- 7.4 the gene product of the open reading frame (orf) ytfP,
- 5 7.5 the pckA gene coding for phosphoenol pyruvate carboxykinase,
- 7.6 the poxB gene coding for pyruvate oxidase,
- 7.7 the dgsA gene coding for the regulator of the phosphotransferase system, and
- 10 7.8 the fruR gene coding for the fructose repressor.

Fig. 1:



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PCT

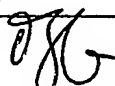
010131 BT

Original (for SUBMISSION) - printed on 05.03.2002 09:26:15 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2002)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	010131 BT

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	13
1-2	line	1-5
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	08 September 2000 (08.09.2000)
1-3-4	Accession Number	DSMZ 13720
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

## FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	YES
0-4-1	Authorized officer	 B. GATINET (0)70/340218

## FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	



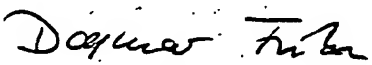
BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

PCT/EP02/02421

INTERNATIONAL FORM

Degussa-Hüls AG  
Kantstr. 2  
33790 Halle

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR: DH5 $\alpha$ /pMAK705	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I. above was accompanied by:  <input checked="" type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable).	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2000-09-08 (Date of the original deposit) <sup>1</sup> .	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH  Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):   Date: 2000-09-12

<sup>1</sup> Where Rule 6.4 (c) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE


PCT/EP02/02421

INTERNATIONAL FORM

Degussa-Hüls AG  
Kantstr. 2  
33790 Halle

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
Identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa-Hüls AG Kantstr. 2 Address: 33790 Halle	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720  Date of the deposit or the transfer <sup>1</sup> : 2000-09-08
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2000-09-08 On that date, the said microorganism was  <input checked="" type="checkbox"/> viable  <input type="checkbox"/> no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED <sup>4</sup>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH  Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):    Date: 2000-09-12

<sup>1</sup> Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

<sup>2</sup> In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

<sup>3</sup> Mark with a cross the applicable box.

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number  
WO 02/081722 A3

(51) International Patent Classification<sup>7</sup>: C12P 13/08,  
13/04 // (C12P 13/08, C12R 1:19)

(21) International Application Number: PCT/EP02/02421

(22) International Filing Date: 6 March 2002 (06.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
101 16 518.8 3 April 2001 (03.04.2001) DE

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1,  
40474 Düsseldorf (DE).

(72) Inventors: RIEPING, Mechthild; Mönkebergstrasse 1,  
33619 Bielefeld (DE). HERMANN, Thomas; Zirkon-  
strasse 8, 33739 Bielefeld (DE).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,

GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,  
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN,  
YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,  
NE, SN, TD, TG).

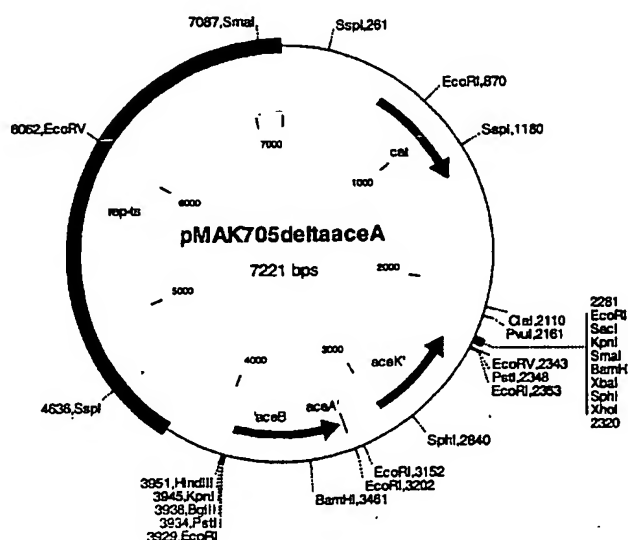
Published:

- with international search report
- with (an) indication(s) in relation to deposited biological  
material furnished under Rule 13bis separately from the  
description

(88) Date of publication of the international search report:  
30 October 2003

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTE-  
RIACEAE THAT CONTAIN AN ATTENUATED *aceA* GENE



(57) Abstract: The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the fol-  
lowing steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino  
acid, in which the *aceA* gene or nucleotide sequences coding therefor are attenuated, in particular are switched off; b) enrichment of  
the L-amino acid in the medium or in the cells of the bacteria; and c) isolation of the L-amino acid.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 02/02421

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12P13/08 C12P13/04 //(C12P13/08,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 368 266 A (TOSAKA OSAMU ET AL) 11 January 1983 (1983-01-11) column 1, line 39 -column 2, line 8 examples 1-3 tables 3-5 claim 1	1-7
Y	EP 0 952 221 A (AJINOMOTO KK) 27 October 1999 (1999-10-27) page 2, line 24 -page 3, line 2 page 6, line 42 -page 7, line 6 claim 1	1-7
Y	EP 0 955 368 A (AJINOMOTO KK) 10 November 1999 (1999-11-10) page 2, line 22-56 page 6, line 9-31 claim 6	1-7
-/-		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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\*A\* document defining the general state of the art which is not considered to be of particular relevance

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\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

20 May 2003

Date of mailing of the international search report

02/06/2003

Name and mailing address of the ISA

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Fax (+31-70) 340-3016

Authorized officer

van de Kamp, M

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/02421

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MATSUOKA M ET AL.: "Isolation, hyperexpression, and sequencing of the aceA gene encoding isocitrate lyase in Escherichia coli" JOURNAL OF BACTERIOLOGY, vol. 170, no. 10, 1988, pages 4528-4536, XP008016923 ISSN: 0021-9193 cited in the application abstract	1-7
A	MICHAL G: "Biochemical pathways: an atlas of biochemistry and molecular biology" 1999, JOHN WILEY & SONS INC. AND SPEKTRUM AKADEMISCHER VERLAG, NEW YORK - HEIDELBERG XP002240819 ISBN: 0-471-33130-9 figures 3.8-2, 3.9-1 figures 4.2-1, 4.5-1 and 4.5-2	1-7
A	KRAEMER R: "Genetic and physiological approaches for the production of amino acids" JOURNAL OF BIOTECHNOLOGY, vol. 45, no. 1, 1996, pages 1-21, XP002178648 ISSN: 0168-1656 the whole document	1-7
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# INTERNATIONAL SEARCH REPORT

Internat'l Application No  
PCT/EP 02/02421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 99 53035 A (ALTMAN ELLIOT ;GOKARN RAVI R (US); EITEMAN MARK A (US); UNIV GEORG) 21 October 1999 (1999-10-21) page 5, line 20-24 examples 4,7,9,10 claims 41,49 figures 1,4</p>	1-7
A	<p>JETTEN M S M ET AL.: "Recent advances in the physiology and genetics of amino acid-producing bacteria." CRC CRITICAL REVIEWS IN BIOTECHNOLOGY, vol. 15, no. 1, 1995, pages 73-103, XP000613291 ISSN: 0738-8551 figure 1 page 83, right-hand column, line 1-36 page 90, left-hand column, line 1 -page 92, left-hand column, line 17</p>	1-7
A	<p>CHUNG T ET AL.: "Glyoxylate bypass operon of Escherichia coli: cloning and determination of the functional map." JOURNAL OF BACTERIOLOGY, vol. 170, no. 1, January 1988 (1988-01), pages 386-392, XP008015355 ISSN: 0021-9193 abstract</p>	1-7
A	<p>SAWERS G: "The anaerobic degradation of L-serine and L-threonine in enterobacteria: networks of pathways and regulatory signals" ARCHIVES OF MICROBIOLOGY, vol. 171, no. 1, 1998, pages 1-5, XP002953871 ISSN: 0302-8933 the whole document</p>	1-7
E	<p>WO 02 081698 A (DEGUSSA) 17 October 2002 (2002-10-17) the whole document page 9, line 21 -page 10, line 22 claim 7</p>	1-7
E	<p>WO 02 081721 A (DEGUSSA) 17 October 2002 (2002-10-17) the whole document page 9, line 24 -page 10, line 25 claim 7</p>	1-7

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Information on patent family members

International Application No

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Information on patent family members

International Application No

PCT/EP 02/02421

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